Amyloplast-Localized SUBSTANDARD STARCH GRAIN4 Protein Influences the Size of Starch Grains in Rice Endosperm

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Starch is a biologically and commercially important polymer of glucose and is synthesized to form starch grains (SGs) inside amylolasts. Cereal endosperm accumulates starch to levels that are more than 90% of the total weight, and most of the intracellular space is occupied by SGs. The size of SGs differs depending on the plant species and is one of the most important factors for industrial applications of starch. However, the molecular machinery that regulates the size of SGs is unknown. In this study, we report a novel rice (Oryza sativa) mutant called substandard starch grain4 (ssg4) that develops enlarged SGs in the endosperm. Enlargement of SGs in ssg4 was also observed in other starch-accumulating tissues such as pollen grains, root caps, and young pericarps. The SSG4 gene was identified by map-based cloning. SSG4 encodes a protein that contains 2,135 amino acid residues and an amino-terminal amyloplast-targeted sequence. SSG4 contains a domain of unknown function4 that is conserved from bacteria to higher plants. Domain of unknown function4-containing proteins with lengths greater than 2,000 amino acid residues are predominant in photosynthetic organisms such as cyanobacteria and higher plants but are minor in proteobacteria. The results of this study suggest that SSG4 is a novel protein that influences the size of SGs. SSG4 will be a useful molecular tool for future starch breeding and biotechnology.

Plastids originated from the endosymbiosis of cyanobacteria and can differentiate into several forms depending on their intracellular functions during the plant life cycle (Sakamoto et al., 2008). The amyloplast is a terminally differentiated plastid responsible for starch synthesis and storage. Starch forms insoluble particles in amylolasts, referred to as starch grains (SGs). SGs are easily visualized by staining with iodine solution, and they can be observed using a light microscope. SGs are observed in storage organs such as seed endosperm, potato (Solanum tuberosum) tubers, and pollen grains. Nonstorage tissues such as endodermis and root caps also contain SGs (Morita, 2010).

Cereal endosperm accumulates high levels of starch in amylolasts. The volume of SGs is approximately the same as the volume of amylolasts that fill most of the intracellular space. SGs in rice (Oryza sativa) endosperm are normally 10 to 20 μm in diameter (Matsushima et al., 2010). One amyloplast contains a single SG that is assembled of several dozen smaller starch granules. Each starch granule is a sharp-edged polyhedron with a typical diameter of 3 to 8 μm. This type of SG is called a compound SG (Tateoka, 1962). For compound SGs, starch granules are assembled (but not fused) to form a single SG, which is easily separated by conventional purification procedures. By contrast, simple SGs contain a single starch granule. Simple SGs are produced in several important crops, such as maize (Zea mays), sorghum (Sorghum bicolor), barley (Hordeum vulgare), and wheat (Triticum aestivum; Tateoka, 1962; Matsushima et al., 2010, 2013).

The size of SGs in cereal endosperm is diverse. Maize and sorghum SGs have a uniform size distribution of approximately 10 μm in diameter (Jane et al., 1994; Matsushima et al., 2010; Ai et al., 2011). In barley and wheat, SGs of two discrete size classes (approximately 15–25 μm and less than 10 μm) coexist in the same cells (Evers, 1973; French, 1984; Jane et al., 1994; Matsushima et al., 2010). In Bromus species, intrageneric size...
variations of SGs are observed in which even phyloge-netic neighbors develop distinctly sized SGs (Matsushima et al., 2013). The size of SGs can be controlled by manipulating the activity of starch synthetic enzymes using transgenic plants or genetic mutants (Gutiérrez et al., 2002; Bustos et al., 2004; Ji et al., 2004; Stahl et al., 2004; Matsushima et al., 2010). However, the molecular mechanism that controls the interspecific size variations of SGs has not been resolved.

The SG occupies most of the amyloplast interior, because the SG is approximately the same size as the amyloplast. The size of amyloplasts may affect the size of SGs, or vice versa. Amyloplasts and chloroplasts both develop from proplastids. The size of chloroplasts is controlled by the chloroplast binary fission division machinery, especially by the ring structures that form at the division sites (Miyagishima, 2011). Proteins involved in the ring structures have been isolated, including Filamenting temperature-sensitive mutantZ (FtsZ), Minicell locuS (MinD), MinE, and ACCUMULATION AND REPLICATIONS OF CHLOROPLAST5 (ARC5).

Arabidopsis (Arabidopsis thaliana) mutants that are defective in these proteins have defects in chloroplast division and contain enlarged and dumbbell-shaped chloroplasts. In contrast to the binary fission of chloroplasts, amyloplasts divide at multiple sites and generate a beads-on-a-string structure (Yun and Kawagoe, 2009). The inhibition of the chloroplast division machinery does not result in enlarged amyloplasts (Yun and Kawagoe, 2009).

We recently developed a rapid method to prepare thin sections of endosperm (Matsushima et al., 2010). Using this method, SGs in mature endosperm can be easily and clearly observed. We performed genetic screening for rice mutants defective in SG morphology and size. One of the isolated mutants, substandard starch grain4 (ssg4), develops enlarged SGs in its endosperm. In this study, we characterized ssg4 phenotypes and identified the responsible gene. SSG4 encodes a protein containing 2,135 amino acid residues and an N-terminal plastid-targeted sequence. The domain of unknown function 490 (DUF490) was found at the C terminus of SSG4, where the ssg4 mutation was located. This suggests that SSG4 is a novel factor that influences the size of SGs and has potential as a molecular tool for starch breeding and biotechnology.

RESULTS

Enlarged SGs in ssg4 Mutant Endosperm

The chalkiness of seeds was a distinguishing phenotype of ssg4 grains when compared with wild-type grains of cv Nipponbare (Fig. 1, A–D). Seed size was slightly smaller in ssg4 than in cv Nipponbare, especially with respect to seed width and depth (Fig. 1E). The iodine-stained thin sections of mature endosperm clearly showed enlarged SGs in ssg4 endosperms (Fig. 1, F–I). Quantification of the areas occupied by SGs in the thin sections showed that SGs were approximately 6-fold larger in ssg4 than in cv Nipponbare (Fig. 1J).

The endosperm is a triploid tissue generated by the fusion of sperm and the binucleate central cell of the female gametophyte (Li and Berger, 2012). Therefore, endosperm has four possible genotypes at one gene locus: AAA, AaA, Aaa, and aaa. We performed reciprocal crosses to obtain two distinct heterozygous seeds of SSG4SSG4ssg4 and SSG4ssg4ssg4. Chalkiness was not observed in the endosperm of either heterozygote (Supplemental Fig. S1). The SG sizes of SSG4SSG4ssg4 and cv Nipponbare seeds did not significantly differ, whereas the SG sizes of the SSG4ssg4ssg4 seeds were slightly larger than those of cv Nipponbare (Fig. 1J). This indicated that two wild-type alleles supplied from the female gametophyte were sufficient for the formation of normal-sized SGs, whereas one copy of the SSG4 allele supplied by the sperm was functional but not sufficient for the formation of normal-sized SGs. We next examined starch accumulation in ssg4 grains. The total amount of starch was lower in ssg4 seeds than in wild-type seeds (Fig. 1K). No significant difference in the gelatinization properties of ssg4 starch compared with wild-type starch was observed; therefore, the structural properties of starch were similar in ssg4 and cv Nipponbare (Table I). This result was consistent with previous work showing that the amylopectin chain-length distribution of ssg4 starch is normal (Matsushima et al., 2010).

The Arabidopsis phosphoglucomutase (pgm) mutant contains small amounts of starch in leaves but exhibits high levels of accumulation of soluble sugars, such as Suc, D-Glc, and D-Fru (Bläsing et al., 2005). This is explained by the defective conversion of photosynthate into starch in pgm1 leaves. Less starch accumulation in ssg4 seeds also might cause the abnormal level of sugar accumulation. We analyzed the soluble sugars in ssg4 and cv Nipponbare seeds by using gas chromatography-time of flight-mass spectrometry. Levels of Suc and D-Glc were much higher in ssg4 seeds than in cv Nipponbare seeds (Supplemental Fig. S2, A and B), while the D-Fru level was less abundant in ssg4 seeds than in cv Nipponbare seeds (Supplemental Fig. S2C).

Rice grains require more than 1 month for full ripening after flowering. During this period, a large number of SGs are developed and fill the endosperm. To investigate when the enlarged SGs were developed in the ssg4 mutant, we focused on early-developing seeds at 3, 5, and 7 d after flowering (DAF). Seed enlargement from 3 to 7 DAF in cv Nipponbare and ssg4 was essentially the same (Fig. 2, A–F). By contrast, the sizes and numbers of SGs from 3 to 7 DAF in cv Nipponbare and ssg4 were different (Fig. 2, G–L). At 3 DAF, most SGs in the ssg4 endosperm were larger than those in cv Nipponbare (Fig. 2, G and J) and occupied an area that was more than 3-fold larger than that occupied by SGs in cv Nipponbare (Fig. 2M). At 7 DAF, the area occupied by SGs was more than 5-fold larger in ssg4 than in cv Nipponbare. When the SGs were assumed to be spherical, the volume of SGs at 7 DAF was approximately 10-fold larger in ssg4 than in cv Nipponbare. The number of SGs showed the opposite pattern to the sizes of SGs (Fig. 2N) and was lower in ssg4 than in cv
Nipponbare at all days tested. At 3 to 7 DAF, the number of SGs in ssg4 was one-third less than the number in cv Nipponbare (Fig. 2N).

We also investigated ssg4 endosperms at 5 DAF by transmission electron microscopy (TEM; Supplemental Fig. S3). Morphologies of ssg4 SGs in TEM images were spherical, like the iodine-stained SGs in Figure 2.

**SG Morphologies in Other Tissues**

Endosperm tissue accumulates the highest levels of starch in rice plants. Other tissues also accumulate SGs, including pollen grains, root caps, and pericarps. We examined SG morphologies in these tissues in ssg4 mutants. Pollen grains were immersed in iodine solution to stain SGs, and many rod-like SGs were visualized in cv Nipponbare pollen grains (Fig. 3A). By contrast, ssg4 SGs in pollen were more spherically shaped (Fig. 3B). In both cases, pollen SGs displayed different morphologies from those of endosperm SGs. When pollen grains were squashed under coverslips, SGs were released and the morphologies were clearer (Fig. 3, C and D). Scanning electron micrographs of the released SGs also showed that the SG morphologies were different in cv Nipponbare and ssg4 (Fig. 3, C and D, insets). Most SGs in

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**Figure 1.** Enlarged SGs of mature endosperm in the ssg4 mutant. A and B, Grains of cv Nipponbare, front and side view images, respectively. Bars = 1 mm. C and D, ssg4 grains, front and side view images, respectively. Bars = 1 mm. E, Quantification of cv Nipponbare and ssg4 seed sizes (n = 30 each). F and G, Iodine-stained thin sections of cv Nipponbare endosperm at low and high magnification, respectively. Bars = 10 μm. H and I, Iodine-stained thin sections of ssg4 endosperm at low and high magnification, respectively. Bars = 10 μm. J, Quantification of the areas occupied by SGs in sections of different genotypes (n = 6 each). K, Quantification of the starch amount in mature seeds expressed as the percentage of weight (n = 3 each). Data are given as means ± se. Statistical comparisons were performed using Welch’s t test; all data were compared with cv Nipponbare (*P < 0.05, **P < 0.01).
Pollen grains of both cv Nipponbare and ssg4 appear to be simple SGs. SGs were slightly larger in ssg4 pollen grains than in cv Nipponbare pollen grains (Fig. 3E). Root caps developed many SGs that were the compound type (Fig. 3, F–I). The pericarp is the wall of the mature ovary, and it surrounds the entire seed. In early-developing rice seeds, many compound SGs developed in the pericarp (Fig. 3, K–N). The SGs in the ssg4 pericarps were more spherical than those in the cv Nipponbare pericarps (Fig. 3, K–N). In root caps and

**Table I. Effects of the ssg4 mutation on the gelatinization properties of starch in endosperm determined by differential scanning calorimetry**

Gelatinization properties of the starch in ssg4 seeds were analyzed by a differential scanning calorimeter. Values are means ± s.e. of three independent determinations. T₀, Tₚ, and Tₑ are onset, peak, and conclusion temperatures, respectively. ΔH is gelatinization enthalpy of starch.

<table>
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<tr>
<th>Plant</th>
<th>T₀</th>
<th>Tₚ</th>
<th>Tₑ</th>
<th>ΔH</th>
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<tr>
<td>cv Nipponbare</td>
<td>53.2 ± 2.6</td>
<td>63.2 ± 0.8</td>
<td>69.2 ± 0.5</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>ssg4</td>
<td>51.6 ± 2.1</td>
<td>61.6 ± 0.4</td>
<td>67.6 ± 0.8</td>
<td>5.8 ± 0.5</td>
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**Figure 2.** SGs in maturing endosperm. A to C, Developing seeds of cv Nipponbare (NP) at 3, 5, and 7 DAF, respectively. Bars = 1 mm. D to F, Developing seeds of ssg4 at 3, 5, and 7 DAF, respectively. Bars = 1 mm. G to I, Iodine-stained thin sections of cv Nipponbare endosperm at 3, 5, and 7 DAF, respectively. Bars = 20 μm. J to L, Iodine-stained thin sections of ssg4 endosperm at 3, 5, and 7 DAF, respectively. Bars = 20 μm. **M**, Quantification of the areas occupied by SGs in sections at 3, 5, and 7 DAF (n = 20 each). **N**, Quantification of the numbers of SGs per 10,000 μm² at 3, 5, and 7 DAF. Data are given as means ± s.e. Statistical comparisons were performed by Welch’s t test; all data were compared with cv Nipponbare (**P < 0.01**).
pericarps, ssg4 SGs were more than 2-fold larger than cv Nipponbare SGs (Fig. 3, J and O). All these results suggest that the ssg4 mutation affects the size of SGs in pollen grains, root caps, pericarps, and endosperm.

The third leaves of ssg4 mutants showed a variegated phenotype (Fig. 4, A and B). We speculated that chloroplasts might also be affected by the ssg4 mutation. To visualize chloroplasts, thin sections of third leaves from young seedlings were double stained with methylene blue and basic fuchsin (Fig. 4, C–F). The cv Nipponbare chloroplasts displayed elongated, lens-like shapes, whereas those of ssg4 were more spherical (Fig. 4, E and F). The areas of chloroplasts were approximately 2-fold larger in ssg4 than in cv Nipponbare (Fig. 4G). These results indicate that the ssg4 mutation affects the size of chloroplasts and amyloplasts. We also investigated ssg4 chloroplasts by TEM to observe the chloroplastic ultrastructures, such as starch granules, grana stacks, and envelope membranes. The size of starch granules in ssg4 chloroplasts were similar to those in cv Nipponbare chloroplasts (Fig. 4, H and I). Grana stacks and envelope membranes were not affected in ssg4 chloroplasts (Fig. 4, J and K). In contrast to the third leaves, ssg4 flag leaves did not show the variegated phenotype (Supplemental Fig. S4, A and B). The shapes of chloroplasts in the flag leaves did not show much difference between cv Nipponbare and ssg4 (Supplemental Fig. S4, C–F). Areas of chloroplasts in the ssg4 flag leaves were a little larger than those in cv Nipponbare, but not to the degree in the third leaves (Supplemental Fig. S4G). TEM showed that the chloroplastic ultrastructures were not affected in the ssg4 flag leaves (Supplemental Fig. S4, H–K).

Figure 3. SG morphologies in pollen grains, root caps, and pericarps. A and B, Iodine-stained pollen grains of cv Nipponbare and ssg4, respectively. Bars = 10 μm. C and D, Released SGs from squashed pollen grains of cv Nipponbare and ssg4, respectively. Bars = 10 μm. Insets show scanning electron micrographs of the released SGs. Bars = 1 μm. E, Quantification of the areas occupied by SGs in pollen grains (n = 30 each). F and G, Iodine-stained thin sections of root caps of cv Nipponbare and ssg4, respectively. Bars = 20 μm. H and I, Magnified images of F and G. Bars = 20 μm. J, Quantification of the areas occupied by SGs in root caps (n = 24 each). K and L, Iodine-stained thin sections of pericarps in 3-DAF seeds of cv Nipponbare and ssg4, respectively. Bars = 10 μm. M and N, Magnified images of K and L. Bars = 10 μm. O, Quantification of the areas occupied by SGs in pericarps (n = 12 each). Data are given as means ± se. Statistical comparisons were performed by Welch’s t test; all data were compared with cv Nipponbare (**P < 0.01).
Genetic Analysis and Map-Based Cloning of the SSG4 Gene

When ssg4 was crossed with cv Nipponbare, approximately half of the pollen grains of the F1 plants had ssg4 phenotypes (Table II). This indicates that ssg4 behaves in a gametophytic manner in pollen grains. The ssg4 phenotype in endosperm tissue was completely penetrant in ssg4 selfed progeny and segregated as a single recessive allele in F2 progeny (Table III). We identified the SSG4 gene using conventional map-based cloning. We mapped the ssg4 mutation within a 62-kb region on chromosome 1 (Fig. 5A). Ten open reading frames are expected in this region according to the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). We identified a base change in the Os1g0179400 gene of the ssg4 mutant. The ssg4 mutant carries a G-to-A transition at position 4,139,234 (The International Rice Genome Sequencing Project [IRGSP] 1.0-based position) on chromosome 1. The G-to-A transition is consistent with an ethyl methanesulfonate-induced mutation. A previously isolated complementary DNA (cDNA) clone of Os1g0179400 (AK063507) encodes a protein containing 1,022 amino acid residues with a DUF490, according to the Pfam database (Punta et al., 2012). SSG4 is similar to the EMBRYO DEFECTIVE2410 (EMB2410) protein in Arabidopsis. Although AK063507 was registered as a full-length cDNA, all other homologous proteins from Arabidopsis, Brachypodium distachyon, and maize contain more than 1,000 additional amino acids at their N termini, compared with the Os1g0179400 protein predicted from AK063507. This raises the possibility that the reported 5' terminus of AK063507 is incorrect and that a longer protein is

Table II. Segregation of ssg4 pollen grains of F1 plants

<table>
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<tr>
<th>Parental Genotype</th>
<th>No. of Wild-Type Pollen Grains</th>
<th>No. of ssg4 Pollen Grains</th>
<th>Total</th>
<th>Percentage of ssg4 Pollen Grains</th>
</tr>
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<tbody>
<tr>
<td>ssg4+/+</td>
<td>59</td>
<td>58</td>
<td>117</td>
<td>50.4</td>
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Figure 4. Chloroplast morphologies in ssg4 third leaves. A and B, Third leaves of cv Nipponbare and ssg4, respectively. Bars = 1 mm. C and D, Thin sections of the third leaves were double stained with methylene blue and basic fuchsin in cv Nipponbare and ssg4, respectively. Bars = 10 μm. E and F, Magnified images of C and D. Bars = 10 μm. G, Quantification of the areas occupied by chloroplasts in third leaves (n = 12 each). Data are given as means ± SD. Statistical comparisons were performed by Welch's t test; all data were compared with cv Nipponbare (**P < 0.01). H and I, TEM images of chloroplasts of cv Nipponbare and ssg4, respectively. Bars = 1 μm. J and K, TEM images of thylakoid and envelope membranes of cv Nipponbare and ssg4, respectively. Bars = 200 nm.
encoded by the real Os1g0179400 full-length cDNA. To investigate this possibility, we performed a 5′ RACE experiment to determine the 5′ end of Os1g0179400. The RACE experiment showed that the 5′ end of Os1g0179400 is far longer than that of AK063507. The new full-length cDNA of Os1g0179400 is derived from 23 exons and the 5′ untranslated region at the 5′ terminus (Fig. 5B). The deduced protein had 2,135 amino acid residues and contains a putative plastid-targeting sequence at the N terminus. For the complementation test, we cloned the genomic sequence of 14,263 nucleotides, starting from the putative N terminus. For the complementation test, we cloned the genomic sequence of 14,263 nucleotides downstream of the stop codon of the Os01g0179400 gene. We could not clone the promoter sequence of SSG4 because it was unstable and caused deletions during plasmid construction. Therefore, we used the maize UBIQUITIN1 promoter to express the Os01g0179400 genomic clone (Himmelbach et al., 2007). The genomic clone was introduced into the mutant, and the transgenic plants that were homozygous for the transgene were isolated and named SSG4. The sizes and morphologies of SGs in transgenic Ubi:SSG4genomic/ssg4 endosperm and pollen grains were very similar to those in cv Nipponbare (Fig. 6). This indicates that the SG phenotypes in endosperm and pollen grains were completely rescued by the transgene. We conclude that Os1g0179400 is the gene responsible for the ssg4 mutation.

SSG4 had a putative plastid-targeting sequence in its N-terminal region (Fig. 5B). Other than the plastid-targeting sequence and DUF490, no other functional domains were identified in the SSG4 protein. Phylogenetic analysis showed that DUF490s from photosynthetic organisms form a different group separate from proteobacterial DUF490s (Fig. 5C). The SSG4 protein is targeted to chloroplasts and has a putative transit peptide at the N-terminal region (Fig. 5B). We used the N-terminal coding region (639 bp) of the SSG4 gene fused to GFP. We used the N-terminal coding region (639 bp) of the SSG4 cDNA instead of the full-length cDNA because the full-length cDNA sequence strongly inhibited bacterial growth and was difficult for plasmid construction. The plasmid construct containing the N terminus of SSG4 fused to GFP was designated SSG4N-GFP. When SSG4N-GFP was transiently expressed in Nicotiana benthamiana leaves, the SSG4N-GFP signals were detected inside chloroplasts, and the patterns were very similar to the stroma-localized GFP (Supplemental Fig. S6). This result indicates that SSG4N-GFP was mainly localized in stroma of chloroplasts.

Expression Patterns of the SSG4 Gene

The expression patterns of SSG4 in various tissues of different developmental stages were investigated using real-time quantitative PCR by three different sets of primers (Supplemental Fig. S5). P1, P2, and P3 primer sets were used to detect the first, middle, and last exons of the SSG4 gene, respectively (Fig. 5B). All tissues except for third leaves were sampled from plants grown in a paddy field. To obtain third leaves, plants were grown in a greenhouse. Real-time quantitative PCR showed that SSG4 was expressed in all tissues examined in both cv Nipponbare and ssg4 (Supplemental Fig. S5A). This suggests that SSG4 is needed at all developmental stages. During early seed development, SSG4 transcripts started to accumulate at 4 DAF in cv Nipponbare, but the accumulation was delayed in ssg4. At 5 to 7 DAF, the expression of SSG4 continued to increase in both cv Nipponbare and ssg4, reaching a high level. In young plants, the third leaves in cv Nipponbare had a high level of SSG4 expression, which was approximately twice as high in ssg4. The higher expression of SSG4 in third leaves compared with the flag leaves in cv Nipponbare may reflect the greater requirements of SSG4 in third leaves. This is consistent with the severe enlargement of chloroplasts in the third leaves compared with the flag leaves in ssg4 (Fig. 4; Supplemental Fig. S4). The expression patterns obtained using the P2 and P3 primer sets were approximately the same as that obtained using the P1 primers (Supplemental Fig. S5, B and C). This indicates that all three primer sets amplified the same cDNA species. Therefore, the long full-length SSG4 cDNA determined in this study should be the dominant cDNA species.

Subcellular Localization of the SSG4 Protein

The target prediction programs TargetP (Emanuelsson et al., 2007) and WoLF PSORT (Nakai and Horton, 2007) predicted that the SSG4 protein is targeted to chloroplasts and has a putative transit peptide at the N terminus. To confirm the chloroplast localization of SSG4, we attempted to construct the SSG4 gene fused with GFP. We used the N-terminal coding region (639 bp) of the SSG4 cDNA instead of the full-length cDNA because the full-length cDNA sequence strongly inhibited bacterial growth and was difficult for plasmid construction. The plasmid construct containing the N terminus of SSG4 fused to GFP was designated SSG4N-GFP. When SSG4N-GFP was transiently expressed in Nicotiana benthamiana leaves, the SSG4N-GFP signals were detected inside chloroplasts, and the patterns were very similar to the stroma-localized GFP (Supplemental Fig. S6). This result indicates that SSG4N-GFP was mainly localized in stroma of chloroplasts.

We constructed stable transgenic rice plants expressing the SSG4N-GFP gene under the control of the maize UBIQUITIN1 promoter. In SSG4N-GFP plants, SSG4N-GFP fluorescence was detected in pollen grains, endosperm, and...
Figure 5. Map-based cloning of the SSG4 gene. A, Fine-mapping of the SSG4 locus on chromosome 1. A total of 229 F2 progeny (458 chromosomes) with homozygous ssg4 alleles were analyzed. The numbers of recombinations that occurred between SSG4 and the molecular markers are indicated. The SSG4 locus was mapped to a 62-kb region between two molecular markers (Marker1169 and Marker13025). This region contains 10 open reading frames (boxes). The ssg4 mutant has a mutation...
pericarps (Fig. 7). In pollen grains, SSG4N-GFP fluorescence was observed as a ring-like structure (Fig. 7, A–F). Differential interference contrast images of pollen showed that the ring-like GFP fluorescence surrounded rod-shaped structures (Fig. 7, E and F), which are likely to be SGs, as their morphologies are consistent with the iodine-stained SGs shown in Figure 3. In developing endosperm and pericarps, SSG4N-GFP colocalized with the amyloplasts, whose interiors contained compound SGs (Fig. 7, G–I). SSG4N-GFP was excluded from the SGs and accumulated in nonstarch areas (Fig. 7, J). In endosperm SGs, each starch granule is compactly assembled, which might prevent the SSG4N-GFP protein from entering the intergranule space. SSG4N-GFP accumulated in the spaces between SGs and amyloplast membranes (Fig. 7, J–L). This space will correspond to the stroma in endosperm amyloplasts. By contrast, SSG4N-GFP fluorescence accumulated in the space between the starch granules in pericarp SGs (Fig. 7, M–O). This suggests that starch granules in pericarp SGs are loosely assembled, which allows SSG4N-GFP to enter the intergranule space. Taken together, these data show that SSG4N-GFP is localized in the amyloplasts of various tissues and suggest that SSG4 is an amyloplast-localized protein with an N-terminal plastid-targeting signal.

Accumulation of Proteins Involved in Chloroplast Division in ss4 Seeds

In rice, the arc5 mutant is the only mutant reported to be defective in chloroplast division. However, the arc5 endosperm does not produce spherical amyloplast with increased diameter, as ss4 does (Yun and Kawagoe, 2009). The proteins involved in chloroplast division (FtsZ1, FtsZ2, MinD, and MinE) accumulated at the same level in ss4 and cv Nipponbare (Supplemental Fig. S7).

Therefore, we speculate that SSG4 is not directly involved in the regulation of plastid division.

Protein Length Diversity of DUF490-Containing Proteins

In the InterPro protein sequence analysis and classification database (Hunter et al., 2012), 4,546 DUF490-containing proteins are registered. Proteins containing DUF490 are found from bacteria to higher plants but not in animals. TamB (for translocation and assembly module B) is a well-characterized DUF490-containing protein in proteobacteria and is responsible for the insertion and assembly of outer membrane proteins (Selkirk et al., 2012). Out of the 4,546 DUF490-containing proteins, proteobacterial proteins predominate and include 3,566 proteins, whereas 166 proteins are registered for cyanobacteria and 41 proteins are registered for Viridiplantae (green algae and land plants). A comparison of the lengths of these DUF490-containing proteins showed that proteins from cyanobacteria and Viridiplantae are clearly longer than those from proteobacteria (Supplemental Fig. S8, A–C). The lengths of most proteobacterial proteins are approximately 1,300 amino acid residues. For example, TamBfs from Citrobacter rodentium, Salmonella enterica, and Escherichia coli are all 1,259 amino acids residues (Selkirk et al., 2012), while the majority of cyanobacterial and Viridiplantae proteins are around 2,000 amino acid residues. The differences in protein length distributions among proteobacteria, cyanobacteria, and Viridiplantae are statistically significant (Steel-Dwass analysis: proteobacteria and cyanobacteria, $P < 0.001$; proteobacteria and Viridiplantae, $P = 0.005$; cyanobacteria and Viridiplantae, $P = 0.542$). Several DUF490-containing proteins of Viridiplantae with around 2,000 amino acid residues are predicted to target plastids. Therefore, the longer DUF490-containing proteins may be needed for photosynthetic organisms and organelles.
DISCUSSION

Regulation of SG Sizes by SSG4

The size of an SG is one of the most important characteristics of starch for industrial applications (Lindeboom et al., 2004). Small starch granules are used to replace fat in food applications, because aqueous dispersions of small starch granules exhibit fat-mimetic properties (Malinski et al., 2003). In maize and cassava (*Manihot esculenta*) crops, larger starch granules are desirable because they improve the final yield after wet-milling purification (Gutiérrez et al., 2002).

In this study, we characterized *ssg4* phenotypes that show enlarged SGs in endosperm, pollen grains, root caps, and pericarps (Figs. 1–3). SSG4 was identified as the gene that influences SG size (Fig. 5). An amino acid substitution from Gly to Ser in DUF490 of SSG4 increased the size of SGs. However, the enlargement of SGs did not result in the direct expansion of starch granules in *ssg4*, because SGs in *ssg4* endosperm are the compound SG type. The information obtained in this study will be applicable to other crops for the production of larger starch granules. For simple SGs, the size of

![Figure 6](image_url)

**Figure 6.** Complementation of the *ssg4* mutant with the genomic clone of the SSG4 gene. The genomic fragment including the SSG4 gene was expressed under the control of the maize UBIQUITIN1 promoter in the *ssg4* mutant background. Two independent transgenic plants (*Ubi:SSG4genomic/ssg4* #5 and #8) were examined using the following tissues: mature endosperm (A–D) and pollen grains (E–H). A and B are low magnification; C and D are high magnification; E and F are images of whole-mount pollen grains; G and H show SGs that were released from squashed pollen grains. Bars = 10 μm.

![Figure 7](image_url)

**Figure 7.** Amyloplast localizations of SSG4N-GFP of various tissues. Confocal and differential interference contrast (DIC) images of SSG4N-GFP transgenic plants are shown. A to C, Whole-mount images of pollen grains. Bar = 10 μm. D to F, Higher magnification images of amyloplasts in pollen grains. Bar = 5 μm. G to I, Endosperm sections obtained by vibratome sectioning. Bar = 10 μm. J to L, Higher magnification images of endosperm sections. Arrowheads indicate the nonstarch regions in which GFPs accumulated. Bar = 5 μm. M to O, Pericarp sections obtained by vibratome sectioning. Bar = 10 μm.
the SG is consistent with the size of starch granules. Therefore, the enlargement of SGs will directly generate larger starch granules. Barley, maize, and sorghum develop simple SGs, and all these species have homologs of SSG4. These homologs have a conserved Gly residue at the mutation site of ssg4 reported in this study, like the wild-type rice cv Nipponbare. Therefore, introduction of this same mutation into these crops or down-regulation of homologs of SSG4 will produce larger starch granules.

A number of mutants defective in starch biosynthetic enzymes of endosperms have been isolated in several plant species (Walker and Merritt, 1969; Jarvi and Eslick, 1975; Satoh and Omura, 1981; Yano et al., 1984; Satoh et al., 2003a, 2003b, 2008; Kang et al., 2005; Fujita et al., 2007, 2009). Some of these mutants exhibit distinct SG morphologies in endosperms compared with those of wild-type plants. Mutations in amylopectin-branching enzyme IIb reduce the size of SGs in rice and maize endosperm (Yano et al., 1985; Li et al., 2007; Matsushima et al., 2010), while the Arabidopsis mutant of starch synthase IV forms one huge starch granule per chloroplast in leaves (Roldán et al., 2007). Starch synthase IV is suggested to be involved in the process of initiation of the starch granule and in the priming of starch synthesis (Szydłowski et al., 2009; D’Hulst and Mérida, 2010). However, the role of starch synthase IV in cereal endosperms has remained unknown so far.

Subcellular Localization of SSG4

The SSG4 N-terminal sequence targeted GFP to the stroma of chloroplasts and amyloplast in various tissues (Fig. 7; Supplemental Fig. S6), while proteomic analysis of cyanobacteria showed that SSG4 cyanobacterial homologs are localized in the outer membrane (Moslavc et al., 2005). The Prediction of Transmembrane Regions and Orientation program (http://www.ch.embnet.org/software/TMPRED_form.html) predicted three transmembrane regions in the SSG4 sequence (amino acids 104–127, 517–540, and 1,463–1,485). The first region had the highest confidence interval. However, the first region should not be a transmembrane domain because it is included in SSG4N-GFP. The latter two regions may be important for the intraplasmatic localization of SSG4 and may target SSG4 to membranes.

Possible Functions of the SSG4 Protein

To date, SSG4 homologs of photosynthetic organisms have not been functionally characterized. Transfer DNA knockout mutations of the Arabidopsis homolog of SSG4 (At2g25660), denoted as emb2410, arrest embryo development at the globular stage (Meinke et al., 2008). Many embryo-defective mutants with the disruption of plastid-targeted proteins have been shown to exhibit the impaired plastid development (Hsu et al., 2010; Bryant et al., 2011). In the case of the Arabidopsis arc1 mutant, the weak allele mutant has smaller, more numerous chloroplasts than the wild type, while the strong transfer DNA insertion allele causes embryo lethality (Kadirjan-Kalbach et al., 2012). ARC1 likely functions in an essential process of plastid development that may be coupled with plastid division. In a similar way, the essential function of At2g25660 for embryogenesis raises the possibility that the function of SSG4 is more involved in plastid development than any direct role for SG size control. The pleiotropic effect of the ssg4 mutation on chloroplast organization in third leaves and the conservation of DUF490-containing proteins in cyanobacteria that do not develop SGs support this idea (Figs. 4 and 5).

Septum-like structures have been suggested to exist between starch granules during the formation of compound SGs (Yun and Kawagoe, 2010). The successive synthesis of septa during plastid division promotes the formation of compound SGs. SGs in endosperms, root caps, and pericarps were the compound type, while SGs in pollen grain were the simple type (Figs. 1–3). The enlargement of SGs by the ssg4 mutation was extreme in compound SGs compared with simple SGs (Fig. 3). Therefore, SSG4 may be related to septum formation and may have some specific roles in the compound nature of dividing plastids. The amino acid substitution in ssg4 is the first nonlethal mutation in DUF490-containing proteins of photosynthetic organisms. Future studies of SSG4 will reveal more detailed information about the function of DUF490 in higher plants.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Rice (Oryza sativa subspecies japonica ‘Nipponbare’ and subspecies indica ‘Kasalath’) were used as wild-type plants. The ssg4 mutant was previously isolated from an ethyl methanesulfonate-treated cv Nipponbare M2 population (Matsushima et al., 2010). We backcrossed the ssg4 mutant with cv Nipponbare, and their progeny with ssg4 phenotypes were used in this study. Rice plants were grown at an experimental paddy field at the Institute of Plant Science and Resources, Okayama University, under natural conditions or at 28°C in a greenhouse.

Characterization of Grain Appearance, Size, and Starch Amount

 Mature dry seeds and maturing young seeds were photographed with a macromicroscope (MVX10; Olympus) and a digital camera (DP72; Olympus). The sizes of grains were measured by vernier caliper. The total amount of starch was measured by enzymatic methods using the Total Starch Assay Kit (Megazyme International).

Thermal Properties of Starch

Dried rice grain was dehulled, crushed with pliers, and hand homogenized using a motor and pestle. The weighed starch (3 mg) was placed in a silver sample cup (560-003; Seiko Instruments), mixed with 9 μL of distilled water, and sealed. Gelatinization properties of the starch were analyzed by a differential scanning calorimeter (DSC-6100; Seiko Instruments). The heating rate was 3°C min⁻¹ over a temperature range of 5°C to 90°C.

Semiquantification of D-Glc, D-Fru, and Suc Levels

Extracts from mature seeds of cv Nipponbare and the ssg4 mutant (equivalent to 5 mg) were subjected to gas chromatography-time of flight-mass
spectrometry as described previously (Kusano et al., 2007). Peaks of r-Gc, d-Fru, and Suc in each analyte were identified by comparing retention indices and the mass spectra of the corresponding authentic standards.

Thin Sections of Technovit 7100 in Endosperm and Staining

For mature dry seeds, approximately 1-mm³ blocks were cut from the center region of the endosperm and fixed in solution containing 5% (v/v) formalin, 5% (v/v) acetic acid, and 50% (v/v) ethanol for at least 12 h at room temperature. For maturing endosperm, approximately 1-mm blocks were cut out from the maturing endosperm at 3, 5, and 7 DAF and fixed in 3% (v/v) glutaraldehyde in 20 mM cacodylate buffer (pH 7.4) for at least 24 h at 4 °C. To observe root caps, the seminal root tips (1 mm) were cut out and fixed in the same buffer as for the maturing endosperm. To observe chloroplasts, the middle region of the leaf was sampled. After fixation, samples were subsequently dehydrated and then embedded in Technovit 7100 resin (Kulzer) as described previously (Matsushima et al., 2010). The embedded samples were cut in 1-µm sections with an ultramicrotome (EM UC7; Leica Microsystems) and diamond knives and then dried on coverslips. To stain SGs, thin sections were stained with 40% diluted Lugol solution (iodine/potassium iodide solution; MP Biomedicals) in deionized water for at least 5 s and subsequently examined with a microscope (AX70; Olympus). Quantifications of amyloplast areas were analyzed with ImageJ 1.46r software (http://rsbweb.nih.gov/ij/). To stain the chloroplast in leaves, the Technovit sections were double stained with 0.026% (w/v) methylene blue and basic fuchsin.

TEM Observation

The middle region of third leaves of seedlings and 5-DAF endosperms were fixed in 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.4) at 4 °C overnight followed by postfixation with 2% (w/v) osmium tetroxide at 4 °C for 3 h. Samples were subsequently dehydrated using a graded ethanol series and infiltrated with propylene oxide. The samples were then embedded in Quetol-651 resin (Nissin EM). The embedded samples were ultrathin sectioned at 70 nm with a diamond knife, and sections were placed on copper grids. They were stained with 2% (w/v) uranyl acetate for 15 min, and then they were secondary stained with lead stain solution for 3 min. The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV. Digital images were taken for 3 min. The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV. Digital images were taken for 3 min. The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV. Digital images were taken for 3 min.

Observation of Pollen Grains

To obtain the iodine-stained mature pollen grains, anthers just before anthesis were disrupted with forceps in the diluted Lugol solution on a glass slide, and the released mature pollen grains were subsequently dehydrated using a graded ethanol series and infiltrated with propylene oxide. The samples were then embedded in Quetol-651 resin (Nissin EM). The embedded samples were ultrathin sectioned at 70 nm with a diamond knife, and sections were placed on copper grids. They were stained with 2% (w/v) uranyl acetate for 15 min, and then they were secondary stained with lead stain solution for 3 min. The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 80 kV. Digital images were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions).

Map-Based Cloning of the SSG4 Gene

For mapping the SSG4 mutant, a genomic fragment containing the Os01g0179400 gene was cloned into plpPKb002, a binary vector for the transformation of cereals (Himmelbach et al., 2007). The genomic fragment is 14,263 nucleotides, starting from the putative first ATG up to 1,299 nucleotides downstream of the stop codon of Os01g0179400 (chromosome 1, 4,127,368–4,141,631). The genomic fragment was amplified separately as two fragments to construct the plasmid. First, the 3' half of the genomic region (4,131,703–4,141,631) was amplified using the following primers: 5'-TACGTC-GACTCGATCTAAATGCGGCCTGTCGACGACG-3' and 5'-TACTCTTCCCTACCCCTCCCGGCTGC-3'. The fragment was cloned into the BamHI and EcoRI sites of the pENTRB2 entry vector (Invitrogen) in the Fusion-Cloning Kit (Clontech). The resulting plasmid is called pENTR-latter. The remaining 5' half of the genomic region (4,131,703–4,133,132) was amplified using the following primers: 5'-AACCAATTCGATGAATTGTCGCTGTCGACGACG-3' and 5'-TACGTC-GACTCGATCTAAATGCGGCCTGTCGACGACG-3'. The fragment was then introduced into the SSG4 mutant using an Agrobacterium tumefaciens-mediated method (Hiei et al., 1994). To obtain the transgenic plants expressing the SSG4 protein fused with GFP, the genomic DNA fragment was amplified and cloned into the pENTR-latter vector (Invitrogen) with the Gateway system. The resulting plasmid, pENTR-full, was used for the LR recombination reaction with the destination vector plPKb002 using the Gateway system (Invitrogen). The resulting plasmid was then introduced into the SSG4 mutant using an Agrobacterium tumefaciens-mediated method (Hiei et al., 1994). To test whether the transgenic plants expressing the SSG4 protein were functional, the plantlets were transferred to the soil. For the GFP gene, the SSG4 N-terminal coding region (303 nucleotides) was amplified by PCR using the full-length cDNA as a template and the following primers: 5'-AAGAACATTGCATGAACTTGTCGCTGTCGACGACG-3' and 5'-TACGTC-GACTCGATCTAAATGCGGCCTGTCGACGACG-3'. The amplified fragments were connected and inserted into the Soll site and SacI sites of the pENTR-latter. The Soll site is located in the vector-derived region, and the SacI site is in the middle of the genomic region. The resulting plasmid, pENTR-full, was used for the LR recombination method with the destination vector plPKb002 using the Gateway system (Invitrogen). The resulting plasmid was then introduced into the SSG4 mutant using an Agrobacterium tumefaciens-mediated method (Hiei et al., 1994).

Phylogenetic Analysis

Sequences containing DUF490 were searched through BLAST in the GenBank/EMBL/DNA Data Bank of Japan (DDBJ) databases and the Munich Information Center for Protein Sequences database (http://mips.helmholtz-muenchen.de/plant/index.jsp). Sequences were aligned with ClustalW (http://www.genome.jp/tools/clustalw/), followed by manual alignment. Trees were constructed on conserved positions of the alignment by clustered protein sequence alignment plants with the neighbor-joining algorithm. The tree was constructed using the MEGA 5.2 with pairwise deletion for gap filling (Tamura et al., 2011). To test inferred phylogeny, we used bootstrap with 1,000 bootstrap replicates.

Expression Pattern of the SSG4 Gene

Different tissues, including developing seeds, anthers, pistils, young panicles, and third and flag leaf blades were sampled. Except for third leaf blades,
A. tumefaciens-Mediated Transient Transformation of N. benthamiana

Suspensions of transformed A. tumefaciens GV3101 bacteria were adjusted to an optical density of 600 nm of 0.6 in MES buffer (10 mM MgCl₂ and 10 mM MES, pH 5.6), and acetosyringone was added to a final concentration of 20 μM. Bacterial suspensions were then maintained at room temperature for 2 to 3 h. Infiltrations were conducted by gently pressing a 1-mL disposable syringe to the abaxial surface of fully expanded leaves that were approximately 3.5 cm wide and slowly depressing the plunger. A sufficient amount of bacterial suspension was used to completely infiltrate the leaves and give a water-soaked appearance. Following the infiltration, plants were maintained in a growth chamber at 25°C with a 12-h/12-h light/dark photoperiod. Leaves were examined by microscopy between 50 and 90 h after infiltration. For chloroplast stroma-localized GFP, plasmid (pL12-GFP) expressing GFP fused to Cor413 chloroplast inner envelope membrane protein1 was used (Okawa et al., 2008). GFP signals were detected using a laser scanning confocal microscope (FV1000; Olympus).

Detection of GFP Signals in Endosperms and Pericarps of SSG4-GFP Transgenic Plants

Developing seeds (3 DAF) without husks were embedded in 5% (w/v) agarose and cross sectioned through the middle portion of the seed in 150-μm-thick sections with a Vibrating Blade Microtome (VT-1200S; Leica Microsystems). The sections were incubated in phosphate-buffered saline, and the samples were examined using the laser scanning confocal microscope (FV1000).

Total Protein Extraction and Immunoblotting

For protein extraction from developing seeds, samples were homogenized in the extraction buffer (10 μL mg⁻¹) consisting of 50 mM Tris-HCl (pH 6.8), 8 M urea, 4% (w/v) SDS, 20% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol (35 μL mg⁻¹) using a plastic homogenizer. After centrifugation at 12,000g for 5 min, proteins were separated by SDS-PAGE on precast 10% to 20% polyacrylamide gels (ATTO). The gels were blotted onto a polyvinylidene fluoride membrane for immunoblotting with antibodies and enhanced chemiluminescence (GE Healthcare). Antibodies were prepared previously (Yun and Kawago, 2009).

The accession number for the SSG4-coding DNA sequence is AB856288 in GenBank/EMBL/DDBJ. All other sequence data used in this article can be found in the GenBank/EMBL/DDBJ databases and the UniProt Knowledgebase.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Grain chalkiness of ss4l and heterozygous mutant seeds.

Supplemental Figure S2. Semiquantification of Suc, d-Glc, and d-Fru levels in cv. Nipponbare and ss4l.

Supplemental Figure S3. TEM images of SGS at 5 DAF.

Supplemental Figure S4. Chloroplast morphologies in ss4l flag leaves.

Supplemental Figure S5. SSG4 expression patterns in various tissues.

Supplemental Figure S6. Stroma localization of SSG4N-GFP in chloroplasts.

Supplemental Figure S7. Accumulation of proteins involved in chloroplast division in ss4l seeds at 7 DAF.

Supplemental Figure S8. Histograms showing the protein length distributions of DUF490-containing proteins.

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